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### Industrial Perspectives on Assays

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*Published in:*

Enzyme Assays: High-throughput Screening, Genetic Selection and Fingerprinting

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*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

2006

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Sonke, T., Duchateau, L., Schipper, D., Euverink, G-J., Wal, S. V. D., Henderickx, H., Bezemer, R., & Vollebregt, A. (2006). Industrial Perspectives on Assays. In J-L. Reymond (Ed.), *Enzyme Assays: High-throughput Screening, Genetic Selection and Fingerprinting* (pp. 95-135). s.n..

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## 4

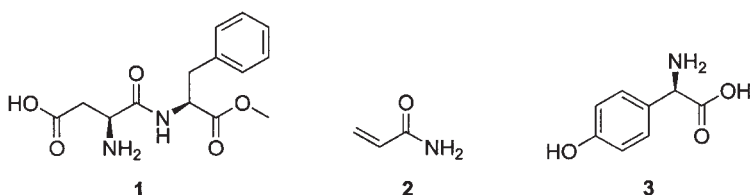
## Industrial Perspectives on Assays

Theo Sonke, Lucien Duchateau, Dick Schipper, Gert-Jan Euverink,  
Sjoerd van der Wal, Huub Henderickx, Roland Bezemer, and Aad Vollebregt

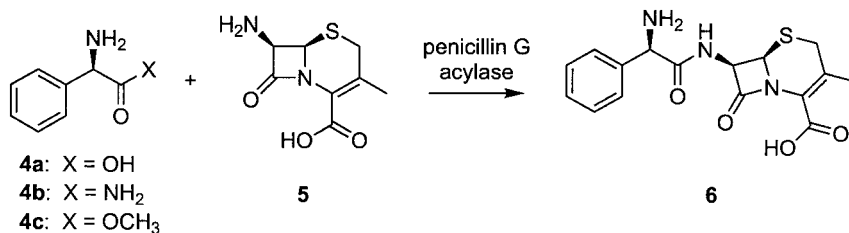
## 4.1

## Introduction

Although microbial cells and enzymes have been used by humans for millennia to produce a variety of foodstuffs, it was not until the twentieth century that biocatalysis was introduced in the chemical industry. Since that time it has grown to become an established technology for chemical manufacturing. Examples of biocatalytic processes currently successfully operated at industrial scale are the production of the low-calorie sweetener aspartame (1) catalyzed by the protease thermolysin [1, 2], the production of the bulk chemical acrylamide (2) using a nitrile hydratase [3, 4], and the production of D-*p*-hydroxyphenylglycine (3) using microbial cells containing a hydantoinase and a carbamoylase [5, 6].



The recent need for more sustainable processes has driven the implementation of biocatalysis in industry further, in many cases by replacing existing chemical processes. An example of such second-generation “green processes” is the chemo-enzymatic synthesis of the semi-synthetic  $\beta$ -lactam antibiotic cephalixin (6). In this new process concept the side-chain D-phenylglycine (4a), activated as amide (4b) or ester (4c), is coupled to the  $\beta$ -lactam nucleus 7-amino-desacetoxycephalosporanic acid (7-ADCA, 5) by a penicillin G acylase (Figure 4.1) [7]. This process fully exploits the advantages of a biocatalyst in an industrial setting. The coupling reaction of activated side-chain and nucleus is done in water at ambient temperature, thereby eliminating the use of halogenated



**Fig. 4.1** Penicillin G acylase-catalyzed synthesis of the semi-synthetic  $\beta$ -lactam antibiotic cephalixin (**6**).

solvents and low temperatures. The strict selectivity of the biocatalyst contributes to the high efficiency and increased sustainability of this chemo-enzymatic process making protection of the two building blocks and deprotection of the coupling product superfluous [8, 9]. Altogether, over 140 processes using enzymes in isolated form or microbial cells have been commercialized up to now, most of which deliver small-scale chiral products for applications in the pharmaceutical sector [10, 11].

Chemical custom manufacturing (CCM) is the industry segment in which the fine chemicals industry produces a compound on demand for a client (usually a pharmaceutical company) in a one-to-one relation (as opposed to multi-client products). In this highly dynamic business area, speed and responsiveness are among the most important key success factors. General timelines for CCM are given in Table 4.1. It can be seen that proof of principle including a sample often needs to be delivered to the customer within just three months.

In CCM the use of biocatalysis for the production of (chiral) fine chemicals is still limited compared with, for instance, resolution via diastereomer crystallization or chromatography, asymmetric organometal catalysis, or conventional

**Table 4.1** Typical timeline for process development within chemical custom manufacturing.

Time	Status	Remark
0	Pharmaceutical customer request	Chemical route exists but usually high costs Quest for alternatives begins
2 weeks	Selection of 2–3 routes	Based on rough cost price estimates Plan and quote to customer After customer's positive response, experimental work begins
2 months	Feasibility demonstrated	Final choice made More accurate cost price calculated
3 months	Lab sample delivered for approval by customer	Based on specs or use tests

chemistry starting from the chiral pool [12, 13]. The main reason for this difference is the long lead time to find and implement a biocatalytic process compared with a chemical process, despite the fact that biocatalysts in general show excellent enantio-, regio- and stereoselectivity, which, in principle, often enables the design of shorter and more cost-efficient processes.

In order to be able to implement a biocatalytic reaction step successfully in such a product, extremely strict timelines to (bio)catalyst identification and subsequent process development need to be met. Although the three months' timeline and approach schematized in Table 4.1 is an average scenario and deviations frequently occur, it clearly demonstrates the challenge for (bio)catalysis in CCM: the time available for (bio)catalyst identification and (small-scale) process development is extremely short.

In this chapter we will deal with the development of enzymatic assays and activity-based screenings from an industrial perspective. We will focus solely on CCM, and try to describe how this specialized branch of the chemical industry must cope with the demanding timelines for biocatalyst identification. In the first part, a number of prerequisites for the ideal screening will be discussed, including one of the most important ones: integral screening time. In the second part of this chapter, these general principles will be exemplified by a number of assay/screening methods developed for DSM's chemical custom manufacturing business unit DSM Pharma Chemicals (DPC).

## 4.2

### Prerequisites for an Effective Biocatalyst Screening in Chemical Custom Manufacturing

Identification of a biocatalyst for a certain chemical conversion requires input of sufficiently high-quality biodiversity to start from. In the last decade tremendous progress has been made in the generation of such biodiversity. Construction of expression libraries directly from environmental DNA is applied in many laboratories as a source of diversity. Use of this so-called metagenome now enables identification of interesting biocatalysts from microorganisms that could not be cultivated in laboratories before, increasing the biodiversity dramatically [14]. The rapidly increasing quantity of genome sequence information, together with improved software tools to predict the function of the encoded proteins, offers another source for new enzymes. This genome sequence information can be transferred into ready-to-screen enzymatic platforms.

Directed evolution is another relatively recent approach to the creation of biodiversity. Numerous formats ranging from simple error-prone polymerase chain reaction (epPCR) [15, 16] of a single gene to shuffling of large families of genes [17, 18] and even whole genomes [19, 20] have been published in the last 10 years to access the desired sequence space. These molecular biology tools have become quite robust, which makes the generation of sufficiently high-quality biodiversity no longer a limiting factor in many biocatalyst identification pro-

grams. This is also true in CCM if the inputs for the screening (for example expression libraries and enzymatic platforms) have been generated proactively to meet the short timelines in this industrial segment.

Screening for biocatalysts has also attracted considerable attention from academia and industry in the last decade, undeniably resulting in significant progress in this field [21, 22]. Nevertheless, the development and implementation of a new screening within the limited time available in CCM is still a significant challenge. The following criteria are pivotal for successful screening in this demanding field.

### **Screening for Real Conversion**

One of the most commonly used statements in screening is “You get what you screen for”. In the open and patent literature, numerous artificial substrates have been described for colorimetric or fluorometric detection of a wide range of enzymatic activities, but use of this type of substrate bears the inherent risk that a biocatalyst identified does not catalyze the desired reaction. This can be caused by steric incompatibility (artificial substrates are often larger than the actual ones) or catalytic incompatibility (certain types of artificial substrates are more active than the actual substrates). Although improved artificial substrates have recently been reported [23], screening for the real conversions still results in the most reliable hits. Furthermore, synthesis of the artificial substrate can be difficult and time consuming, which will delay the outcome of the screening, thereby making it even harder to identify the desired biocatalyst on time.

### **Robust and Reliable**

The ideal screening is robust and reliable. It must not suffer from matrix effects, such as small differences in pH, salt concentration, and residual growth medium. Furthermore, interexperimental differences should be small. We noticed that the robustness of screening protocols sharply decreases with increasing numbers of handling (e.g. centrifugation, pipetting, and filtration). Therefore, simple screening protocols are preferred whenever possible. Altogether, the screening method must clearly discriminate true positive clones without delivering a large number of false positives.

### **Quantitative**

To be able to discriminate positive clones the applied screening protocol must result in a clear change of measured response. However, different types of projects require different levels of accuracy. Screening a (metagenomic) library for new biocatalysts, for example, places less strict requirements on accuracy than

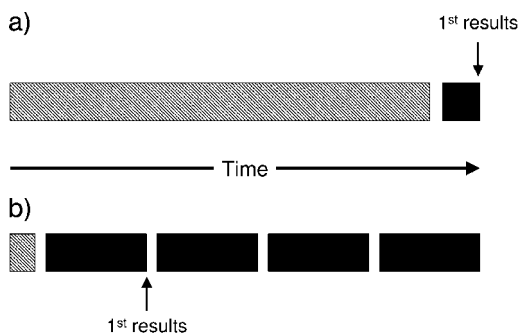
screening in a directed evolution program to improve the catalytic performance of an enzyme. In the first example, the scenario is more or less black and white – the few positive clones that are expected should give a signal that is sufficiently different from the background signal. The exact intensity of the signal of the positive clones is not very useful, however, since clones containing the same gene will often have a different genetic constitution that can severely influence the expression of the genes on the insert fragment. Therefore, an assay that delivers semi-quantitative results is sufficient for this type of screening. In the case of a directed evolution project for improved enzymes the situation is different. In this case, the enzymatic reaction is tuned so that the conversion with the wild-type clones amounts to about 10% conversion. Consequently, these wild-type clones will already give a (low) signal, requiring more quantitative screening methods. The fact that all clones to be screened contain the (mutated) gene(s) of interest in an identical genetic constitution is another reason why quantitative screenings are advantageous in this case. As the development of highly quantitative screenings can be time consuming, the accuracy aimed for should be tuned to the needs of the specific application.

### **Sensitive**

As the most reliable results are obtained by mimicking industrial conditions in the screening reaction as far as possible, substrate concentrations are usually high (> 50 mM). Consequently, the sensitivity of the analytical method is typically not an issue. Where the substrates or products are poorly soluble in water the situation is different, and a sensitive analytical method is required. Using mass spectrometry (MS) positive clones can be identified starting from substrate concentrations as low as 0.5 mM. Addition of a cosolvent enabling higher substrate concentrations is another possibility, assuming that it is not interfering with the screening.

### **Integral Screening Time**

This is the most important factor for screenings in CCM. It can be defined as the time needed from the start of the screening development to the generation of the first screening results. As will have become clear from the introductory section, only screening methods with a short integral screening time are compliant with CCM timelines. At first glance, ultra-high-throughput screening (UHTS, in this chapter defined as a screening with a throughput of > 10<sup>4</sup> samples a day) formats look suitable for application within CCM as they are characterized by an extremely short analysis time per sample. However, this is certainly not true in all cases, especially when the development of UHTS methods is time consuming (Figure 4.2).



**Fig. 4.2** Graphical representation of the effect of a short integral screening time on the time 1<sup>st</sup> results are generated. (a) An ultra-high-throughput screening with a long development time (typical for reader assays without colorimetric or fluorometric model substrates). (b) A generic assay that combines

a much shorter development time with lower throughput in the actual assay (typical for instrumental assays like flow-injection NMR). The gray boxes represent the development time for the assay, and the black boxes the time needed for the actual measurements of all samples in a screening.

Reader assays (both UV/Vis and fluorescence) are appropriate, in principle, for use in UHTS. A 96-well microtiter plate can be measured in less than 30 s, leading to a theoretical throughput of  $10^4$ – $10^5$  samples a day in the case of an end-point measurement. Unfortunately, this throughput can only be achieved with simple screening protocols, which are only possible when the conversion of substrate into product leads to strong changes in molar extinction coefficient or fluorescence. As this is quite often not the case with the actual substrates and/or products and use of artificial substrates is undesirable, derivatization of substrates and/or products into highly absorbing or fluorescent compounds is necessary as an additional step. These derivatization reactions not only lead to much more complicated screening protocols and consequently longer screening times (less throughput), but also to much longer development times as they require careful tuning of the derivatization conditions (e.g. reagent concentration, temperature, pH, and reaction time) and solutions for matrix effects. Overall, this will result in a relative long integral screening time (Figure 4.2a), which makes such a screening method less attractive for applications in CCM.

Figure 4.2b shows a situation that is better suited for use in CCM. In this case, implementation of a screening method with very short development time and sufficient throughput (not necessarily ultra high throughput) generates the first screening results in a shorter period of time, leading to quicker hit identification and start of application research as well as sample preparation for the customer. The characteristics needed (short development time and sufficient throughput) are offered by generic assays, as for example based on nuclear magnetic resonance (NMR) or MS. These types of assays are characterized by the fact that they generate distinct signals for many chemical functionalities enabling straightforward differentiation between substrate and product of the enzymatic reaction to be screened. Combined with their relatively good tolerance

to fluctuations in the matrix, these instrumental assay techniques are quite powerful.

A different approach is the proactive development of less generic assays for frequently requested chemical group conversions, as for example the synthesis of amino acids or for reactions producing ammonia as a side-product. In this case the challenge is to find reagents that are specific for these molecules and generate a clearly distinguishable signal in the derivatization reaction. Furthermore, this derivatization reaction must not be interfered by other chemical groups in these molecules, which will be different in each customer's request.

In the next sections of this chapter examples will be discussed from each of these CCM compliant screening approaches.

### 4.3

#### **CCM Compliant Screening Methods Based on Optical Spectroscopy (UV/Vis and Fluorescence)**

As stated in the previous section, reader assays are characterized by a very short measuring time per sample. However, often this does not translate into a short integral screening time as the product formed in the screening reaction lacks a chromophore or fluorophore and consequently needs to be chemically derivatized before it can be detected in a reader with sufficient sensitivity and selectivity. In other cases the product of the screening reaction can be detected in a reader by a coupled enzymatic assay. In such an assay an enzymatic reaction cascade leads to the formation of a reader detectable compound (for instance  $\text{NAD}^+/\text{NADH}$ ). In both approaches, the overall screening protocols are quite laborious and their development is often time consuming.

Nevertheless, reader assays can be successfully applied within the CCM context, for instance when the formation of the (side)-products results in a strong change in molar extinction coefficient (Section 4.3.1), or when the (side)-products formed belong to a category of compounds for which a derivatization method or a coupled enzymatic assay has been developed beforehand (Section 4.3.2).

#### 4.3.1

##### **Optical Spectroscopic Methods Based on the Spectral Properties of the Product Itself**

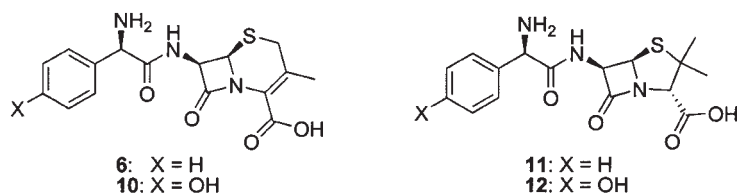
The possibility of implementing screening methods based solely on changes in spectral properties upon conversion of substrate into product is small. Nevertheless, the great potential of this method justifies the efforts that are needed to investigate whether it is possible. Screening requests for transformations of molecules that contain a suitable chromophoric or fluorophoric group can best be started by recording the optical (UV/Vis/fluorescence) spectra of substrate and product. In this way it can be established whether the product absorbs and/or



emits light of a specific wavelength. By including the appropriate blanks in these first experiments, interference of other compounds in the matrix (e.g. the reaction buffer and cell-derived compounds) can be assessed. If these experiments have a positive outcome, a simple screening procedure can be developed in a few days.

#### 4.3.1.1 Example: Isolation of the D-*p*-Hydroxyphenylglycine Aminotransferase Gene [24]

One of the process concepts for the production of enantiomerically pure  $\alpha$ -H- $\alpha$ -amino acids is the conversion of  $\alpha$ -keto acids to the desired amino acids by aminotransferases [25, 26]. For the production of the D-enantiomers of  $\alpha$ -H- $\alpha$ -amino acids, the “standard” aminotransferases require equimolar amounts of D-Ala, D-Glu, or D-Asp as amino donor, which makes this process less attractive as these compounds are rather expensive. A more attractive aminotransferase process to enantiomerically pure D- $\alpha$ -H- $\alpha$ -amino acids is possible with an enzyme that can use cheaply available L-Glu (8) and/or L-Asp as amino donor instead of their D-congeners. Such a stereoinverting aminotransferase had been described before in relation to the production of D-*p*-hydroxyphenylglycine (3) and D-phenylglycine (4a, Figure 4.1) [27, 28]. These amino acids are produced as side-chains in the manufacture of semi-synthetic  $\beta$ -lactam antibiotics such as cephalexin (6), cephadroxy (10), ampicillin (11), and amoxycillin (12) [9, 29]. The reaction catalyzed by this aminotransferase (HpgAT) is shown in Figure 4.3.



We decided to identify the HpgAT gene by screening bacterial expression libraries including that of *Pseudomonas putida* NCIMB 12565, which had been reported to contain HpgAT activity [27]. In this case neither a PCR-based approach nor the synthesis of the complete gene were possible, as the sequence of the HpgAT gene had not been published. Recording of the optical spectra of D-*p*-hydroxyphenylglycine (3) and *p*-hydroxyphenylglyoxylate (9) showed that the latter compound has a specific absorbance at about 300–350 nm (Figure 4.4).

Based on this observation, we designed the following simple screening strategy.

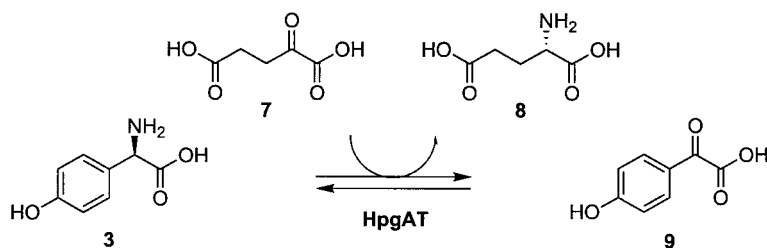


Fig. 4.3 Reaction catalyzed by the stereoinverting D-*p*-hydroxyphenylglycine aminotransferase (HpgAT) from *P. putida* NCIMB 12565.

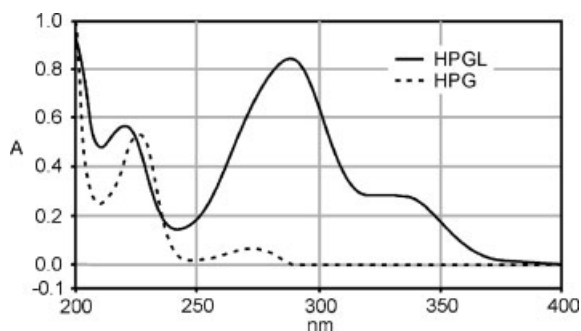
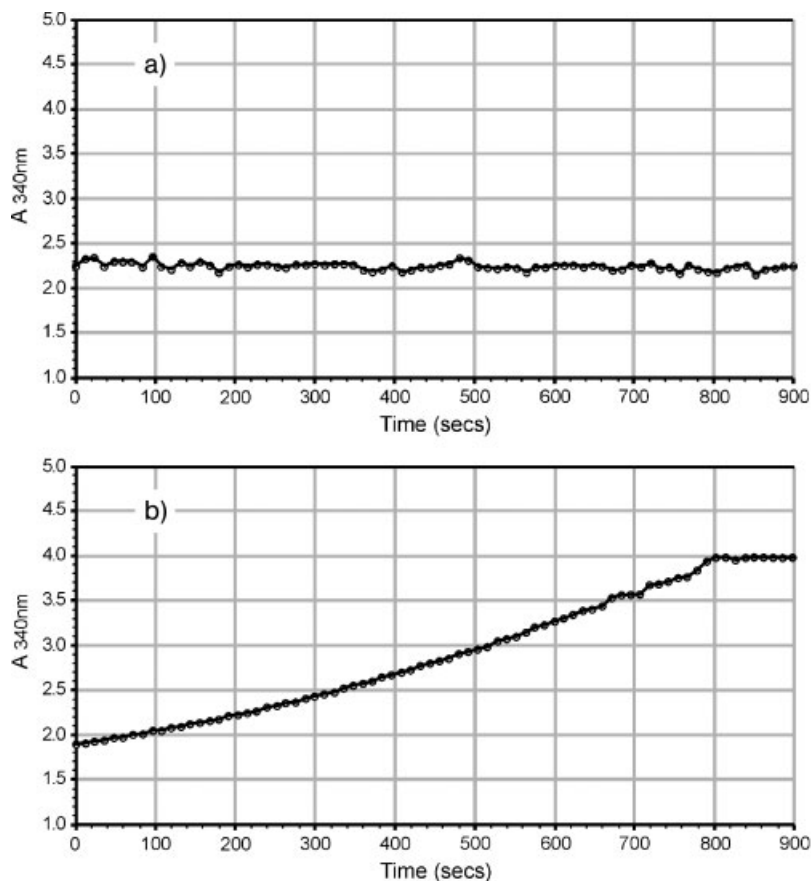


Fig. 4.4 Optical spectra of *p*-hydroxyphenylglycine (HPG, dashed line) and *p*-hydroxyphenylglyoxylate (HPGL, black line) in 45 mM potassium phosphate buffer, pH 7.0. Concentration of both compounds was 55  $\mu$ M.

#### Procedure 4.1: UV Screening of Aminotransferase Reactions

Colonies from a gene library were cultivated in microtiter plates containing 150  $\mu$ L of a rich medium containing the appropriate antibiotic. After 16–20 h at 28°C, cells were harvested by centrifugation, washed once with 50 mM potassium phosphate buffer, pH 7.0, and resuspended in 180  $\mu$ L reaction mix (100 mM potassium phosphate, pH 7.0, 15 mM  $\alpha$ -ketoglutarate (7), 0.1 mM pyridoxal-phosphate (PLP) and 0.5% v/v Triton X-100). The screening reaction was started by addition of D-*p*-hydroxyphenylglycine (3) to a final concentration of 5 mM, after which the  $A_{340\text{nm}}$  in each well was monitored for 20 min using an Optimax microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

As expected, clones containing the desired HpgAT gene showed significant increase in  $A_{340\text{nm}}$  relative to negative wells, as can be seen in Figure 4.5. Integral screening time in this specific case was less than one month due to the very short development time and modest analysis time (20 minutes per 96-well microtiter plate).



**Fig. 4.5** Typical progress curves of (a) a negative *E. coli* clone and (b) an *E. coli* clone containing the HpgAT gene from *P. putida* ATCC NCIMB 12565.

#### 4.3.2

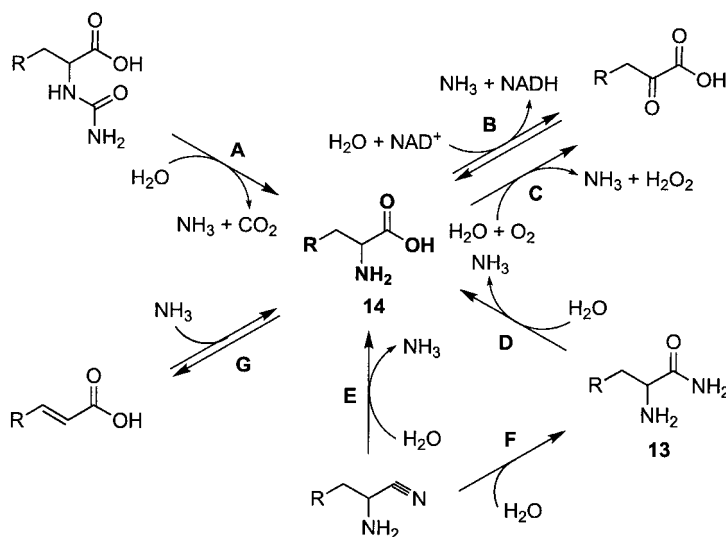
##### Optical Spectroscopic Methods Based on Follow-up Conversion of Product

As the majority of the industrially relevant conversions are not accompanied by clear changes in optical signal, direct reader-based assays are frequently not an option. In many cases, however, a reader assay can still be developed by linking the actual screening reaction to a subsequent conversion that leads to a signal-generating compound. This can be realized in various ways. In a first approach, a chemical reagent or complexing agent reacts with a functional group of the (side)-products. As these derivatization reactions often require harsher conditions (especially pH) than the enzyme reaction to be screened, these types of assays are typically end-point measurements. Interference of the measurement by other compounds that contain the same functional group as the product is the

major complicating factor in these derivatization-based reader assays. A second approach is based on the subsequent conversion of one of the (side)-products of the screening reaction in an enzymatic reaction cascade, in which the final step is a conversion of a reader-detectable compound (for instance  $\text{NAD}^+/\text{NADH}$ ). The major advantage of this second approach is the higher selectivity of the enzyme compared with the chemical derivatization reagent, thereby strongly reducing the number of potentially interfering compounds. Furthermore, the mild conditions needed for the enzymatic cascade more often enable measurements in a kinetic mode.

Although possible in principle, the long development time of these kinds of optical spectroscopic assays hampers their application in CCM. This is due to the large number of parameters that have to be optimized and tuned, such as concentration of the reactants and conditions (pH, temperature, and reaction time) of the different steps as well as their order. Proactive development of robust reader assays for a number of frequently encountered types of molecules ("group assays") can solve this problem and makes these potentially attractive assays CCM timelines compliant.

This approach is further elaborated in the following examples relating to synthetic routes for enantiomerically pure amino acids, which are important chemical building blocks for the pharmaceutical and agrochemical industry [30]. A significant number of the CCM market requests fall within this category. A large number of different chemo-enzymatic process concepts for the production of enantiomerically pure amino acids (Figure 4.6) have been developed and



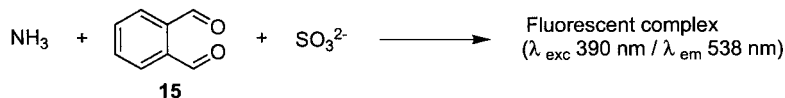
**Fig. 4.6** Schematic overview of different enzymatic reactions to enantiomerically pure amino acids. (A) carbamoylase; (B) amino acid dehydrogenase; (C) amino acid oxidase; (D) amidase; (E) nitrilase; (F) nitrile hydratase; (G) ammonia lyase.

commercialized [31], and ammonia is involved in the majority of these concepts either as side-product (amidases, carbamoylases, nitrilases, or amino acid oxidases) or as substrate (for instance when using amino acid dehydrogenases and ammonia lyases). We set out to develop reader assays for amino acids as well as for ammonia and primarily focused on amidases as these are excellent tools for the production of enantiopure  $\alpha$ -H- and  $\alpha,\alpha$ -disubstituted  $\alpha$ -amino acids [30].

During development of the assays for ammonia, we noticed consistently high backgrounds when crude extracts or cell suspensions of microorganisms were used. This is caused by the growth media, which often contain yeast extract and peptone. Growth of microorganisms in these media involves the production of ammonia from the peptides and amino acids, as these media contain a surplus of nitrogen over carbon. Therefore, it may be necessary to harvest the cells by centrifugation followed by washing with a suitable buffer before starting the actual screening in order to get a sufficiently low background.

#### 4.3.2.1 Example: Fluorometric Detection of Amidase Activity by *o*-Phthalaldehyde/Sulfite Derivatization of Ammonia

One of the reader assays we developed for the quantitative determination of ammonia is based on the derivatization reaction of ammonia with *o*-phthalaldehyde (15) and sulfite, which results in a fluorescent derivatization product (Figure 4.7). The exact structure of this fluorescent product is not known.



**Fig. 4.7** *o*-Phthalaldehyde/sulfite derivatization reaction of ammonia leading to a fluorescent product.

We modified the original flow-injection method [32] into an analytical procedure that can be run in an automated pipetting robot in combination with a microtiter plate fluorescence reader instrument. Using this hardware combination large numbers of enzymatic samples can be screened. The method shows considerable selectivity for ammonia over amino acids and amino acid amides, which makes the method also suitable for monitoring conversion in amidase reactions.

The *o*-phthalaldehyde/sulfite assay was successfully applied in the screening of an epPCR mutant library of a bacterial amidase gene. This screening targeted mutants with improved activity towards a specific  $\alpha$ -H- $\alpha$ -amino acid amide (13, Figure 4.6), without affecting this biocat's absolute enantioselectivity and good process stability.

**Procedure 4.2: Amidase Assay by Spectroscopic Ammonia Detection**

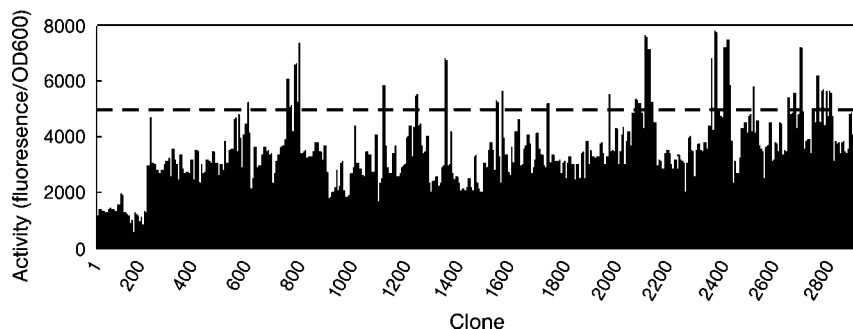
*E. coli* colonies containing the mutant library were cultivated in 96-wells microtiter plates containing 150  $\mu\text{L}$  of a rich medium supplemented with the appropriate antibiotic and inducer. After 18 h at 37 °C, 50  $\mu\text{L}$  cell suspension was mixed with 50  $\mu\text{L}$  of a 20% racemic  $\alpha$ -H- $\alpha$ -amino acid amide solution (pH 8). After 2 h of incubation at 55 °C, the reaction was stopped by adding 200  $\mu\text{L}$  1 M  $\text{H}_3\text{PO}_4$ , after which the microtiter plates were stored at 4 °C before ammonia was assayed. The stopped assay mixture and calibration solutions (*vide infra*) were diluted by mixing 20  $\mu\text{L}$  with 250  $\mu\text{L}$  0.1 M  $\text{H}_3\text{BO}_3$ , pH 9.5. From this mixture, 10  $\mu\text{L}$  was mixed with 45  $\mu\text{L}$  of a 50 mM *o*-phthalaldehyde solution (in 25% methanol, 75% 0.53 M  $\text{H}_3\text{BO}_3$ , pH 9.5) and 45  $\mu\text{L}$  50 mM  $\text{Na}_2\text{SO}_3$  in a 384-well microtiter plate. After 30 min at 55 °C, fluorescence was determined (excitation at 390 nm, emission at 538 nm) in a Fluorstar fluorimeter (BMG laboratories GmbH, Offenburg, Germany). As the signal obtained after this incubation at 55 °C still slightly increased in time, it was essential to measure all plates after the same derivatization time.

For quantification it is a prerequisite that the calibration samples resemble the gene library samples as much as possible. We achieve this by imitating a realistic amidase reaction in the same biological matrix as present in the screened library samples (that is, as conversion proceeds we get increasing amino acid and ammonia concentrations and correspondingly equimolar decreasing amino acid amide concentrations). We call this a complementary calibration procedure. All calibration samples were prepared in 0.67 M  $\text{H}_3\text{PO}_4$  and subjected to the same procedure as the mutant library samples.

To establish the standard deviation of this screening method, the whole screening protocol was executed with a few microtiter plates with *E. coli* cells containing the wild-type amidase gene. The standard deviation of the enzyme activity was less than 15%.

In this specific screening, 30 microtiter plates each containing 96 different random clones resulted in a dataset from 2880 enzyme assays. The absolute data values (Figure 4.8) were corrected for the amount of cells in the assay by dividing the fluorescence by the optical density ( $\text{OD}_{600\text{nm}}$ ) of the cultures.

The resulting specific activities were compared with the activity of the wild-type clones, which resulted in the identification of 41 possible hits for further testing. These mutants were rescreened on a somewhat larger scale. In this second round eight positive mutants were confirmed. Chiral high-performance liquid chromatography (HPLC) analysis of the mutants proved that the enantioselectivity of the amidase was not lost in the mutants.

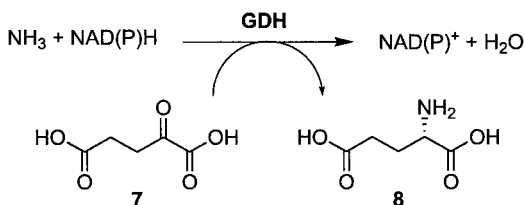


**Fig. 4.8** Data of an *o*-phthalaldehyde/sulfite screening: activities of 2880 clones from an amidase epPCR library normalized for the cell density of the cultures ( $OD_{600nm}$ ). Wild-type level is shown as a dashed line.

#### 4.3.2.2 Example: Colorimetric Detection of Amidase Activity by Detection of Ammonia via Glutamate Dehydrogenase-coupled Assay [33]

A second reader assay we developed to measure the ammonia concentration in high-throughput mode is based on the enzymatic oxidation of NADH by glutamate dehydrogenase (EC 1.4.1.3) [34]. In this reaction glutamate dehydrogenase converts its substrates,  $\alpha$ -ketoglutarate (**7**) and ammonia, into L-Glu (**8**) thereby oxidizing NAD(P)H to NAD(P)<sup>+</sup> (see Figure 4.9). The oxidation of NAD(P)H can be followed in a spectrophotometer at 340 nm or in a fluorimeter (excitation at 339 nm, emission at 460 nm). If the concentration of ammonia to be determined is lower than the concentration of  $\alpha$ -ketoglutarate and NAD(P)H, the decrease in NAD(P)H is directly related to the concentration of ammonia in the sample. It can be calculated using the extinction coefficient of NAD(P)H at 340 nm,  $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ .

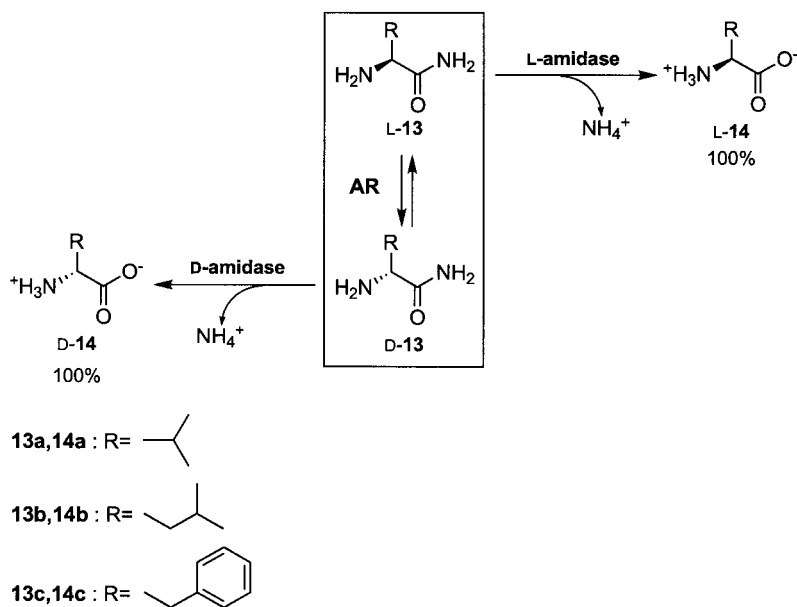
As glutamate dehydrogenase is very specific for ammonia, high concentrations of amino acids and amino acid amides do not interfere with the measurements. This makes this assay very suitable for the quantification of ammonia in samples from different enzymatic conversions as depicted in Figure 4.6, as long as neither NAD(P)<sup>+</sup> nor the reduced form are cofactors in such reactions. Another advantage of this method is the stable signal obtained after the glutamate



**Fig. 4.9** The glutamate dehydrogenase (GDH) reaction, coupling the amount of ammonia in a sample to oxidation of NAD(P)H.

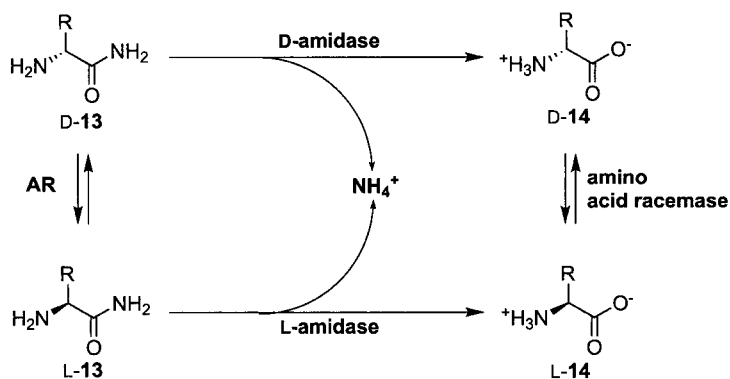
dehydrogenase reaction has gone to completion. This makes the glutamate dehydrogenase incubation step less vulnerable than the *o*-phthalaldehyde (**15**)/sulfite derivatization method described in Section 4.3.2.1. Since screening projects for biocatalysts are usually carried out with crude extracts or cell suspensions of microorganisms, these suspensions may contain high NADH oxidase activity. This will lead to an overestimation of the ammonia concentration because NADH is also oxidized by these enzymes. To prevent this, NADH oxidase activity (as well as other disturbing enzyme activities that may be present in this ammonia assay), can be inactivated by acid treatment of cells, debris and/or proteins, followed by removal of the precipitate via centrifugation, before assaying the ammonia concentration. In many cases, this inactivation will not require an additional pipetting step as the addition of the acid also stops the actual screening reaction, which is a step in many screening protocols (especially for improved enzyme variants) anyhow. Furthermore, this acid treatment will remove all particulates from the screening reaction mixture, preventing noise in the spectrophotometric assay.

We used the glutamate dehydrogenase ammonia assay in a screening project to identify amino acid amide racemases (ARs) [33]. In conjunction with an enantioselective amidase, these enzymes enable the synthesis of enantiomerically pure  $\alpha$ -H- $\alpha$ -amino acids (**14**) from the corresponding racemic  $\alpha$ -H- $\alpha$ -amino acid amides



**Fig. 4.10** Dynamic kinetic resolution (DKR) of  $\alpha$ -H- $\alpha$ -amino acid amides by combining an enantioselective amidase with an amino acid amide racemase (AR), leading to 100% theoretical yield of the desired amino acid enantiomer. The AR reaction is boxed.



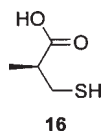


**Fig. 4.11** Schematic representation of possible reaction pathways using a D-a-H-a-amino acid amide as screening substrate for an amino acid amide racemase (AR). Besides the desired pathway via the AR and L-amidase, hits identified may alternatively contain a D-amidase, potentially together with an

amino acid racemase. Both pathways lead to formation of equimolar amounts of L-a-H-a-amino acid and ammonia, which is subsequently detected using the glutamate dehydrogenase assay after the screening reaction has been stopped with phosphoric acid.

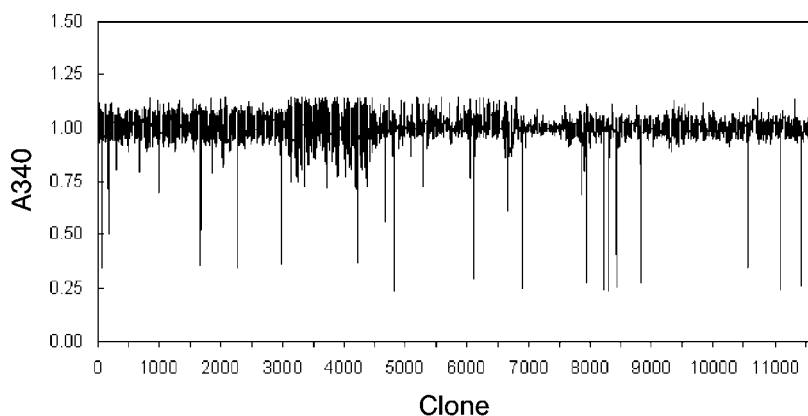
(13) in a dynamic kinetic resolution (DKR) with 100% theoretical yield (Figure 4.10). Without ARs this amidase process is a kinetic resolution with an inherent maximum yield of the desired amino acid stereoisomer of only 50%.

The method for this screening for ARs is shown in Figure 4.11. Expression libraries in *E. coli* are incubated with a D-a-H-a-amino acid amide (D-13) as substrate, which is converted into the corresponding L-a-H-a-amino acid amide (L-13) by AR-positive *E. coli* clones only. As the assay mixture also contains the strictly selective L-aminopeptidase from *Pseudomonas putida* ATCC 12633 [30, 35], the L-a-H-a-amino acid amide formed is hydrolyzed to the L-a-H-a-amino acid (L-14) under formation of ammonia, which will subsequently be determined using the glutamate dehydrogenase assay. As can be seen in Figure 4.11, this screening approach will not only identify AR-positive clones, but also D-selective or nonselective amidase-containing clones, since they will directly hydrolyze the D-a-H-a-amino acid amide screening substrate. Therefore, more specific follow-up assays were needed to distinguish D-amidase from AR-positive clones. This was done on an HPLC system in which all four components of the reaction mixture, namely the a-H-a-amino acid amide (13) and a-H-a-amino acid (14) enantiomers, were quantified after derivatization with o-phthalaldehyde (15) in combination with D-3-mercapto-2-methylpropionic acid (16) as chiral reagent [36].



### Procedure 4.3: Enzyme-coupled Assay for Amino Acid Amide Racemase via NADH Detection

The actual AR screening was executed as follows. Bacterial expression libraries in *E. coli* were cultivated in 96-well microtiter plates containing 200  $\mu\text{L}$  of a rich medium with the appropriate antibiotic. The cultures were grown at 700 rpm and 28–30°C for 2 days, before cells were harvested by centrifugation (10 min at 1500 $\times g$ ), washed twice with 50 mM Tris–HCl buffer, pH 7.5, and resuspended in 50  $\mu\text{L}$  of 50 mM Tris–HCl buffer, pH 7.5. The screening reaction was started by the addition of 50  $\mu\text{L}$  of substrate solution containing a mixture of 140 mM D-valine amide (D-13a), D-leucine amide (D-13b) and D-phenylalanine amide (D-13c) each in 50 mM Tris–HCl buffer, pH 7.5 and 2 mM  $\text{MnCl}_2$ . After 20 h incubation in a microtiter plate shaker at 30°C and 700 rpm, 2  $\mu\text{L}$  of help solution containing the L-aminopeptidase from *P. putida* ATCC 12633 [30, 33] was added to be sure that all L- $\alpha$ -H- $\alpha$ -amino acid amide (L-13) formed in the screening reaction and not yet converted by the endogenous *E. coli* aminopeptidase, was converted into ammonia and L- $\alpha$ -H- $\alpha$ -amino acid (L-14). After an additional 1.5 h incubation at 30°C, all reactions were stopped by the addition of 100  $\mu\text{L}$  of 0.15 M HCl. After removal of the cell debris by centrifugation (10 min at 1500 $\times g$ ), 7  $\mu\text{L}$  of the clear supernatant was transferred to new microtiter plates containing 93  $\mu\text{L}$  of glutamate dehydrogenase (GDH) reagent per well. This GDH reagent contained (per 100 mL) 43 mg of NADH, 116 mg of  $\alpha$ -ketoglutarate (7), 11.8 mg of ADP and 1200 U of GDH (Sigma, St. Louis, MO, USA) in 150 mM Tris–HCl buffer, pH 8.0. After 15 min incubation at 37°C, potential positive clones were determined by measuring the  $A_{340\text{nm}}$  in each well using a Spectramax 250 plus



**Fig. 4.12** Data of a glutamate dehydrogenase screening: decrease of  $A_{340\text{nm}}$  of 11 000 clones from a gene library of *O. anthropi* expressed in *E. coli*. Absolute values obtained have been normalized to the median of each microtiter plate.

microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA). The  $A_{340\text{nm}}$  values obtained in the AR screening of the *Ochrobactrum anthropi* IA gene library are shown in Figure 4.12.

In order to reduce plate-to-plate variation within the  $A_{340\text{nm}}$  data, the following calculation method was used to identify positive hits. From each 96 data points from a 96-well microtiter plate the average  $A_{340\text{nm}}$  and standard deviation were calculated.

Subsequently, the standard deviation was multiplied by a factor of 3 and subtracted from the average. The resulting number was considered as the cut-off value. Clones with an  $A_{340\text{nm}}$  lower than this cut-off value were regarded as potentially positives and selected for further screening. In this way the effect of random noise and plate-to-plate variation was diminished.

Based on this criterion, 32 potential positive clones were selected out of the 11272 clones screened. In the follow-up assays, 22 of the 32 potential positives showed clear  $\alpha$ -H- $\alpha$ -amino acid formation, one of which contained the desired AR gene, as this clone clearly showed L-leucine (L-14b) formation from D-leucine amide (D-13b). The other 21 clones expressed no AR but were most likely either D-selective or nonselective amidases.

#### 4.3.2.3 Example: Colorimetric Detection of Amino Amidase Activity Using $\text{Cu}^{2+}$ as Sensor for Amino Acids [37]

Besides assays based on ammonia detection, we also developed reader assays for the detection of reactions involving amino acids. One of the requirements for such an assay was that it had to be applicable in screening of amino amidases (amidases requiring an  $\alpha$ -amino group for activity). This implied that formation of a small amount of  $\alpha$ -amino acid (product) in a matrix with a usually much higher concentration of  $\alpha$ -amino acid amide (substrate) had to result in a clearly detectable change of spectral or fluorescent properties, in spite of the fact

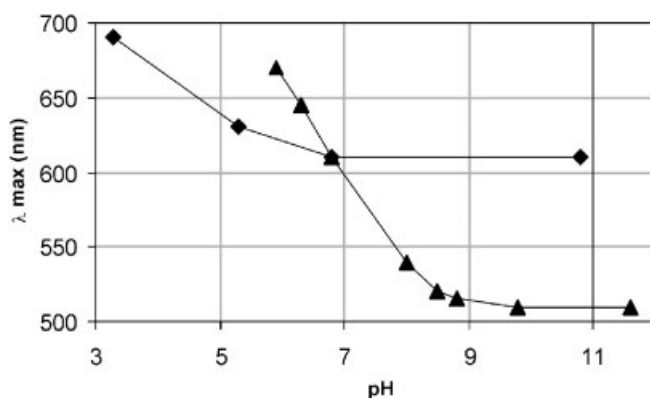
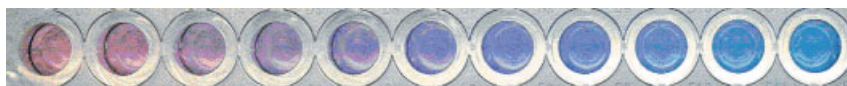


Fig. 4.13 Influence of pH on  $\lambda_{\text{max}}$  of the  $\text{Cu}^{2+}$  complex of valine amide (triangles) and valine (diamonds).

that both compounds contain a primary amine group. Furthermore, interference of the assay by ammonia, which is formed as side-product in equimolar amounts, had to be excluded.

Recently we described a method for the determination of amino amidase activity that meets these requirements [37]. This method is based on the use of  $\text{Cu}^{2+}$  alone as metal sensor, which distinguishes this method from earlier methods that use  $\text{Cu}^{2+}$  or  $\text{Ni}^{2+}$  as metal sensors in conjunction with a fluorescent macrocyclic metal chelator [38, 39]. It appears that the copper complexes of an  $\alpha$ -amino acid and an  $\alpha$ -amino acid amide have different spectral properties at alkaline pH (Figure 4.13).

Below pH 8,  $\alpha$ -amino acid amides form bidentate complexes with  $\text{Cu}^{2+}$ , wherein two Cu-O and two Cu-N bonds are present, resulting in a blue complex ( $\lambda_{\text{max}}$  620–680 nm). At a pH higher than 8, a violet-red complex is formed ( $\lambda_{\text{max}}$  510 nm). The structures of both complexes (the blue as well as the violet-red one) have been described before [40]. The  $\text{Cu}^{2+}$  complex of  $\alpha$ -amino acids, on the other hand, stays blue upon a pH change from 3.3 to 10.8, although there is a shift in  $\lambda_{\text{max}}$  from 690 to 620 nm [37]. The above observations were the basis for the newly developed amino amidase assay method, as this activity will then be indicated by a color change from violet-red ( $\alpha$ -amino acid amide) to blue ( $\alpha$ -amino acid) provided that the pH of the color assay mixture is higher than 8 (Figure 4.14).



**Fig. 4.14** Colors of complementary calibration solutions of  $\alpha$ -methylvaline amide and  $\alpha$ -methylvaline after complexation with  $\text{Cu}^{2+}$ . Color changes from violet-red (outside left well containing 43 mM  $\alpha$ -methylvaline amide) to blue (outside right well containing 43 mM  $\alpha$ -methylvaline and 43 mM ammonia). From left to right, wells contain an increasing concentration of  $\alpha$ -methylvaline and ammonia (4.3 mM per well), and an equi-

molar decreasing concentration of  $\alpha$ -methylvaline amide.

(Reprinted from *Analytical Biochemistry*, Vol. 330, A. L. L. Duchateau, M. G. Hillemans-Crombach, A. van Duijnhoven, R. Reiss, T. Sonke, A colorimetric method for determination of amino amidase activity, pp. 362–364, Copyright (2004), with permission from Elsevier).

We successfully applied this assay method in monitoring amino amidase activity in a large number of fractions resulting from enzyme purification studies.  $\alpha$ -Methylvaline amide (**17a**) was used as substrate, which was converted into  $\alpha$ -methylvaline (**18a**) by the desired amino amidase (Figure 4.15).

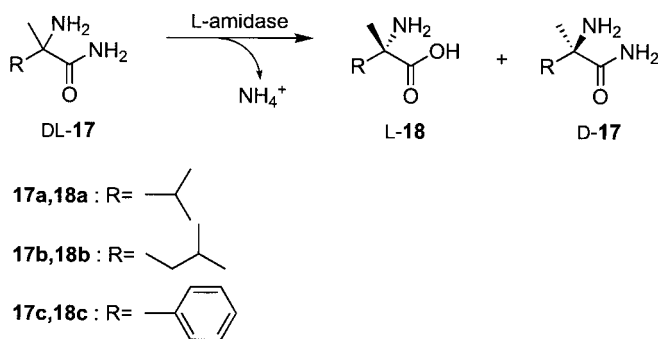


Fig. 4.15 Conversion of DL- $\alpha$ , $\alpha$ -disubstituted  $\alpha$ -amino acid amides by an L-amidase.

#### Procedure 4.4: Colorimetric Amino Amidase Assay Using $\text{Cu}^{2+}$

An aliquot of 50  $\mu\text{L}$  of each fraction was mixed with 100  $\mu\text{L}$  of a 130 mM DL- $\alpha$ -methylvaline amide solution, pH 8.0, and 50  $\mu\text{L}$  400 mM HEPES-KOH, pH 8.0 buffer. After 1 h incubation at 37  $^\circ\text{C}$ , the amino amidase reaction was stopped by the addition of 100  $\mu\text{L}$  1 M  $\text{H}_2\text{SO}_4$ . After removal of particulates by centrifugation, 70  $\mu\text{L}$  of the clear stopped reaction samples were added to 74  $\mu\text{L}$  of an aqueous 20 mM  $\text{CuSO}_4$  solution followed by the addition of 56  $\mu\text{L}$  of a 43:5 mixture of 0.4 M borate buffer pH 9.4 and 10 M KOH. After mixing for 10 s, the absorbance was determined at 620 nm in an Optimax microtiter plate reader (Molecular Devices).

Quantification of the  $\alpha$ -methylvaline formed was possible using a complementary calibration curve approach (Section 4.3.2.1). In this specific case such curve was prepared by mixing increasing amounts of a solution containing 130 mM ammonia and 130 mM  $\alpha$ -methylvaline with correspondingly decreasing amounts of a 130 mM  $\alpha$ -methylvaline amide solution (total concentration amino acid and amino acid amide: 130 mM). Just before measurement, 220  $\mu\text{L}$  of each calibration solution was mixed with 220  $\mu\text{L}$  1 M  $\text{H}_2\text{SO}_4$  and 220  $\mu\text{L}$  0.2 M HEPES-KOH, pH 9.0 to obtain the identical matrix as in the stopped reaction mixtures.

The results obtained showed that fractions containing the desired amino amidase were easily distinguishable by this simple spectrophotometric assay. Furthermore, validation of this  $\text{Cu}^{2+}$  method by measuring the amino amidase-catalyzed conversion of  $\alpha$ -methylvaline amide by this method and HPLC/MS showed that the reader assay delivers quantitative data [37].

## 4.4

### CCM Compliant Screening Methods Based on Generic Instrumental Assays

The short integral screening time required in CCM can also be met by use of generic assays. These assays, which are mostly instrumental, combine extremely

broad applicability with short development time. Therefore, the introduction of these methods can in most cases be done reactively after receiving a question from a customer. This clearly distinguishes them from assay methods requiring proactive development to be compliant with CCM timelines, as is the case with reader assays in which chemical derivatization or enzymatic cascade reactions (Section 4.3.2) are needed to visualize the product. Generic instrumental assays, for instance, are based on NMR and MS

#### 4.4.1

##### **Flow-injection NMR as Analytical Tool in High-throughput Screening for Enzymatic Activity**

NMR spectroscopy is a well-established analytical technique that can yield structural and quantitative information about molecules present in solution. Originally, it was mainly applied in the synthetic field and for physicochemical research; nowadays there are numerous new applications in biology and medicine. The historical drawbacks of low sensitivity and overlapping signals have been overcome by the use of higher field strengths with their intrinsically higher sensitivity and better dispersion of resonances. Recently, the advent of so-called cryo-probeheads again increased the sensitivity by a factor of four.

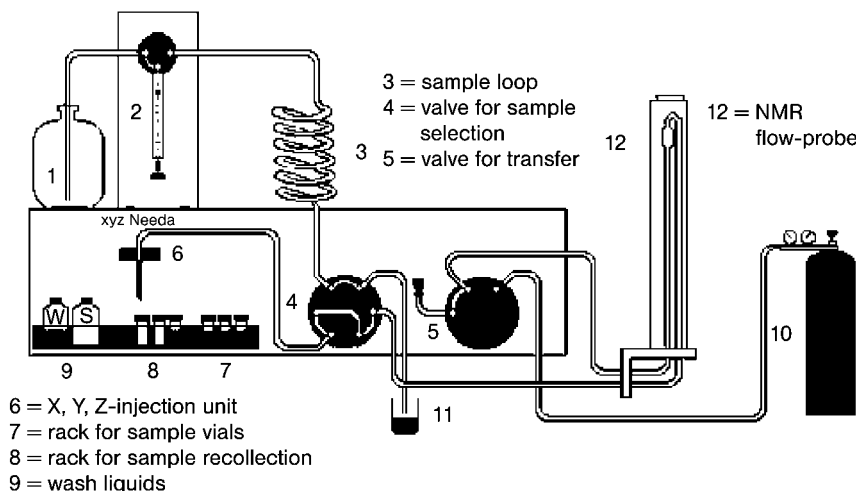
Minimal sample pretreatment and better capability of water suppression also make samples of biological origin amenable for routine analysis. In enzymatic conversions substrate and product are usually different chemical entities with different magnetic properties, so they yield different NMR spectra. Due to the quantitative nature of NMR spectroscopy, conversions can easily be monitored, measuring both substrate and product.

The availability of classical sample changers makes recording of about 100 spectra per day possible. To increase the number of measurements per day, different methods of sample introduction have been developed and the application of flow-through probes has increased the number by one order of magnitude, so that  $^1\text{H}$ -NMR spectroscopy can now be applied to medium/high-throughput screening. In the literature, screening in drug discovery and development, combinatorial chemistry libraries and body fluids is well described [41–45].

##### **4.4.1.1 History**

Convinced by the potential of NMR spectroscopy in the analysis of large series of similar samples, until then limited in our practice to 60 per night, we were thinking about increasing the throughput by alternative sample introduction techniques. Liquid Chromatography/NMR (LC/NMR) had been developed in the late 1980s and further developed in the 1990s and the use of sample loops made it possible to store HPLC fractions in those loops and later introduce and measure stored fractions in a flow-through probe. In 1995 we carried out a first trial at the Bruker site with biological samples, manually introduced into the sample loops. Using the software developed for LC/NMR, it turned out to be

1 = bottle for transport liquid  
2 = single syringe diluter



**Fig. 4.16** Schematic overview of the BEST NMR system based on the Gilson XL215 autosampler.

possible to measure those samples sequentially. Although the speed at that time was still limited due to practical problems of the equipment (thin capillaries restricting the flow), the test was successful in that it proved to be possible to automatically introduce and measure NMR spectra of samples for which the only treatment had been the addition of some  $D_2O$  as a lock solvent [46].

Bruker biospin continued the development, supported by our real-life samples and suggestions. They published together with the Nicholson group in open literature about the principle in 1997 and soon thereafter commercialized their product (Bruker Efficient Sample Transport (BEST) NMR) using a Gilson 215 as a liquid handler and sample changer for vials and well plates [47].

Our first flow-through system (500 MHz) in 1998 was able to change and measure a sample in 3.5 min, which meant a total of 400 samples per day, already 4 times more than the classical sample changers could deliver. Further improvement, by optimizing each step in the process, turned out to be possible in the years thereafter to about 1 min per sample. A crucial element in this was the use of a heatable transfer capillary, which, after careful calibration, made it possible to get the sample to the desired temperature before introduction to the flow-cell, so that the normal temperature equilibration of 4–5 min could be skipped and the measurement started immediately.

For a more detailed description of the principles of flow-through systems on the market the reader is referred to the review of Stockman et al. [45]. A schematic overview of the BEST NMR set-up based on the Gilson XL215 autosampler is given in Figure 4.16.

#### 4.4.1.2 Current Practice

There are several reasons why NMR, and in particular flow-injection NMR, is suitable for screening large libraries for new or improved biocatalysts. These are explained below.

##### **Direct Measurement of Both Substrate and Product**

No artificial substrates are needed for detection purposes. As indicated earlier, this prevents erroneous conclusions, as the artificial substrate is not representative of the aimed substrate, especially in studies to improve the catalytic properties of a specific enzyme. Derivatization reactions are also unnecessary, thus obviating the need for additional steps to the screening.

##### **Intrinsically Quantitative**

Substrate and product in most cases have similar NMR properties, such as relaxation behavior, as long as the size of the molecule does not change dramatically. As a consequence the response factor, which is unity under controlled conditions, also applies under screening conditions.

##### **Short Development Time**

Finding out whether a particular (enzymatic) conversion can be detected by NMR is easily done by measuring the spectra of the intended substrate and product under the expected conversion conditions. This will take less than 1 h. If successful, the actual screening can start, if not, conditions like pH or temperature can be changed to see if the desired separation of the resonances can be obtained. In general it is not necessary to separate all the resonances of the substrate and the product, since one is sufficient.

Where no product is available yet, simulation of the NMR spectrum by specific computer programs gives an indication whether the screening in principle would be successful. In those screenings, manual inspection of the individual spectra is needed to find actual positive hits. This is easily done by scrolling through the spectra at the expected region of interest.

##### **No Calibration Necessary**

Because substrate and product generally have similar molecular weights, integrals of substrate and product resonances can be compared more or less quantitatively, without applying the normally needed long relaxation delay (about 30 s between scans). This way, the actual measuring time can be limited and in practice can be made the same as the time needed for the preparation of the next sample.



### No Critical Sample Pretreatment

The standard sample pretreatment is centrifugation, sampling the supernatant with a robotized system (e.g. 350  $\mu\text{L}$ ) into a deep-well plate, and adding a constant amount (50  $\mu\text{L}$ ) of a solution containing  $\text{D}_2\text{O}$ , EDTA (50 mM), and a known amount of maleic acid. In this way, a lock signal is supplied, and the metal ions, present from the cell-free extracts, are chelated to prevent broadening of the resonances. Moreover, addition of this solution supplies an internal standard to monitor unexpected disappearance of the substrate. In the case of screening for new biocatalysts, it is generally not necessary to stop the screening reaction, for any positive hit is welcome, irrespective of the response in a specific incubation time. When the screening has been set up to improve the enzyme, the reaction should be stopped, for example by adding acid or base, at a moment that the reaction of the wild-type enzyme is around 10% of conversion. In that case improvements can easily be detected.

### Relatively Matrix-independent

NMR spectra are in general not very sensitive to small changes in sample conditions. Most substrate incubations are done between pH 5 and 7, where the influence of pH on the NMR spectrum is normally negligible. If the reaction is stopped by the addition of acid or base, care must be taken that the resulting pH is far enough from the  $\text{pK}_a$  values of the ionizable groups in the molecule, that is below pH 1 or above pH 12.

### No Reference Material Necessary

To observe the conversion of a substrate to an expected product, it is best to have a sample of the product available. If this is not possible, as pointed out under “short development time”, the spectra have to be inspected visually to find the resonances of the product. Once a positive hit has been found, the resonance positions of the product are known and the remainder of the spectra can be evaluated automatically.

### Screening for Multiple Substrates/Products Possible

With flow-injection NMR it is possible to screen more than one substrate to be converted at a time. The boundary condition for this is that each substrate and product has at least one signal that does not overlap with the others, so that conversions for each substrate can be calculated. By measuring the individual spectra from potential substrates, clever combinations can be chosen. In our practice, up to four different substrates have been analyzed for conversion in one round of screening, saving time and effort needed for both the substrate in-

cubation and the measurement. The template for the calculation of the conversions can easily be adapted to report the conversions for individual substrates.

### Unexpected Conversions Detectable

Another advantage of NMR spectroscopy is that, being a heuristic technique *par excellence*, it is not only the expected conversion that can be measured, but also conversions that were not intended. These can be conversions of other functional groups present or follow-up reactions of primary products.

### Robust

NMR spectroscopy in general is a technique without direct interaction between the sample and the instrument during the measurement. There is no wear and no direct contact between the sample and measuring device. In practice, therefore, the downtime is very low. As long as the cryogenic liquids are replenished, the instrument will be in operation. If an electronic device fails, replacement with that item from the manufacturer's stock will solve the problem. For the flow-injection NMR system a specific area that can be problematic is the flow through the capillaries and the flow-cell. As the inlet capillary is thinnest (0.5 mm diameter), possible clogging occurs either there or in the injection port. In those cases, reversal of the flow and flushing with sufficient amounts of cleaning liquid will help. Taking care that centrifugation of the microtiter plates is done correctly and the injection needle is adjusted correctly (so that the needle does not touch the debris present) will in practice prevent clogging.

#### 4.4.1.3 Practical Aspects

To operate the flow-through system routinely, a number of issues need to be dealt with.

### Temperature Equilibration

As most enzyme assays are water-based, a number of spectroscopic problems associated with NMR measurements in water (i.e. temperature equilibration and suppression of the huge water resonance) have to be solved. The temperature of NMR probes is normally actively stabilized at a few degrees above room temperature. As a consequence, a new sample from the sample changer has to equilibrate from room temperature to the temperature of the probehead. This takes about 4–5 min. By preheating the sample on the fly before entering the flow-cell, this time can be saved, thus increasing the number of samples measured. This preheating is done in the capillary between the liquid handler and

the probehead. Bruker developed a heatable transfer capillary that, after careful calibration, does the job. This calibration must be done with actual samples and using the intended dispensing speed on the fly of a series of measurements. When the calibration is correct, the sample will be at the desired temperature and the measurement can start immediately after sample introduction.

### **Adjustment of Field Homogeneity**

In contrast to classical NMR tubes, the flow-cell has a constant volume and wall thickness. Furthermore, the measuring coil is fixed to the outside of the cell. Consequently, each sample experiences the same surroundings, which results in an equal homogeneity of the magnetic field for all samples. In practice this means that the magnetic homogeneity after initial adjustment does not need to be changed or at most by minor adjustment of the Z and Z2 shims. These minor adjustments can be done during the dummy scans (scans without data acquisition, needed to acquire a steady state).

### **Water Suppression**

Water suppression in automation is not trivial. Success is dependent on shimming and stable temperature. The advantage of screening with flow-injection NMR is, as mentioned earlier, that samples are very similar and that the environment inside the probe is more or less constant. In practice this means that initial shimming and determining the correct offset is sufficient to reach a good level of water suppression. Software manipulation of the data can cosmetically improve the spectrum, so that automated processing is possible. If resonances of interest are (too) close to the water resonance, increasing or decreasing the sample temperature will shift the residual water resonance to a more favorable position.

### **Sensitivity and Detection Limit**

The detection limit on a Bruker 500 MHz machine is in the higher micromolar range. By taking more scans this can be lowered, but then the throughput rapidly decreases. When screening for new biocatalysts, substrate concentrations are used that mimic industrial application conditions whenever possible, which corresponds to concentrations of more than 50 mM. That means that in the case of a conversion of 1%, 0.5 mM should be detectable, which in practice is possible. Only when the resonances of the product are highly overlapped by those of background compounds, conditions should be optimized (pH, temperature) to allow detection.

### Back Mixing

An essential prerequisite for applying flow-injection NMR in general is that samples do not mix in the flow-cell. Separation of samples is accomplished by adding cleaning segments and small gaps with air to the sample train that is injected. The amount of back mixing is influenced by factors such as aspiration and dispensing speed, amount of cleaning liquid, number and size of trails, and composition of sample. Some samples can be very sticky, such as solutions containing sugars and polyols. Trial and error with different conditions will allow optimization of the method to be used. When screening large libraries (e.g. 10000 samples or more) for a specific enzymatic activity, speed is limiting. Two factors balance the overall speed possible: sample preparation and data acquisition. The total acquisition time should be equal to the time needed for the preparation of the next sample. This time plus the time that is needed for the actual dispensing of the sample then determines the repetition time. In the case of sufficient sensitivity, sample preparation and sample dispensing become limiting. However, only a few positive hits at most are to be expected and mixing becomes less critical and some mixing can be tolerated. Even 10% of mixing is acceptable, because samples will then report 10% of the activity of the previous sample following a hit and can therefore easily be recognized. An automatic routine in a spreadsheet program can also correct for carryover from the previous experiment.

Samples can be recovered, but this limits the speed to an unacceptable low level for screening purposes.

### Automation (Data Acquisition)

To handle a large number of samples, the NMR manufacturers provide standard software so that the operation of a flow probe does not differ from that of a classical sample changer [45].

### Automation (Data Processing and Handling)

To keep track of the fast acquisition of NMR data, further processing of the data (Fourier transformation, phase correction, baseline correction and integration) needs to be completely automated. Modern NMR software supplies commands that can do automatic phase- and baseline correction that can also be implemented in macros. One method is to run such a macro each time a measurement has finished, the other, more reliable way is to determine the correct phase settings for the first experiment of a series (for instance a microtiter plate) and to run the processing for the series with these settings after the measurement of a microtiter plate has been finished. Automatic baseline correction is easiest when, after initial baseline correction of the complete spectrum, selected regions of interest are corrected with optimized parameters for a specific type of region. Integrals can be set

automatically once they have been determined for a single experiment. In practice the settings are constant for all similar experiments.

To calculate conversions, integrals are put into a text file, which can then be imported into a spreadsheet on a separate computer. There, calculations can be performed by macros that can easily be adapted for individual experiments. For evaluation purposes, a graphical representation of the conversion in a microtiter plate (see Figure 4.20) and a list sorted on highest conversion can be added to a report.

### Cleaning

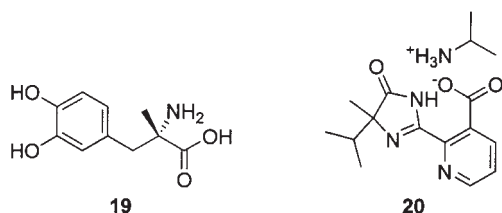
As crude enzyme preparations contain some residual biomass even after centrifugation, precautions have to be taken to prevent microbial growth in the flow system. Adding sodium azide to the cleaning and pushing liquids and flushing the last sample with one or more dummy samples with cleaning liquid is sufficient for routine operation. Regular cleaning with surfactant solution, alcohol, ammonia, and diluted nitric acid is done to remove traces of biomass and sticky components.

### Capacity

NMR machines can run more or less unattended for weeks, as long as the regular filling intervals for liquid nitrogen and helium are maintained. The Gilson 215 liquid handler can contain 10 microtiter plates and most of the screenings are done in 96-well format. With a repetition time of 1 min, new samples have to be added every 16 h, which during the weekends has to be carried out four times when large screenings are done. Switching to a 384-well format, composed of four 96-well plates, limits this number to one at most. Deep-well plates of this format can contain 360  $\mu\text{L}$  liquid, which is sufficient for operation with a 4 mm flow-cell. The use of a 3 mm cryo flow-cell can be done with 160  $\mu\text{L}$  and can thus limit the amount of sample needed.

#### 4.4.1.4 Example: Screening of a Bacterial Expression Library for Amidase-containing Clones

As well as  $\alpha$ -H- $\alpha$ -amino acids,  $\alpha,\alpha$ -disubstituted  $\alpha$ -amino acids are also a group of industrial relevance, as is exemplified by the use of L- $\alpha$ -methyl-3,4-dihydroxyphenylalanine (L- $\alpha$ -methyldopa, **19**) as an antihypertensive drug [48, 49] and  $\alpha$ -methylvaline (**18a**, Figure 4.15) as an intermediate for the herbicide Arsenal (**20**), currently marketed as its isopropylamine salt by BASF, and related herbicides [50].

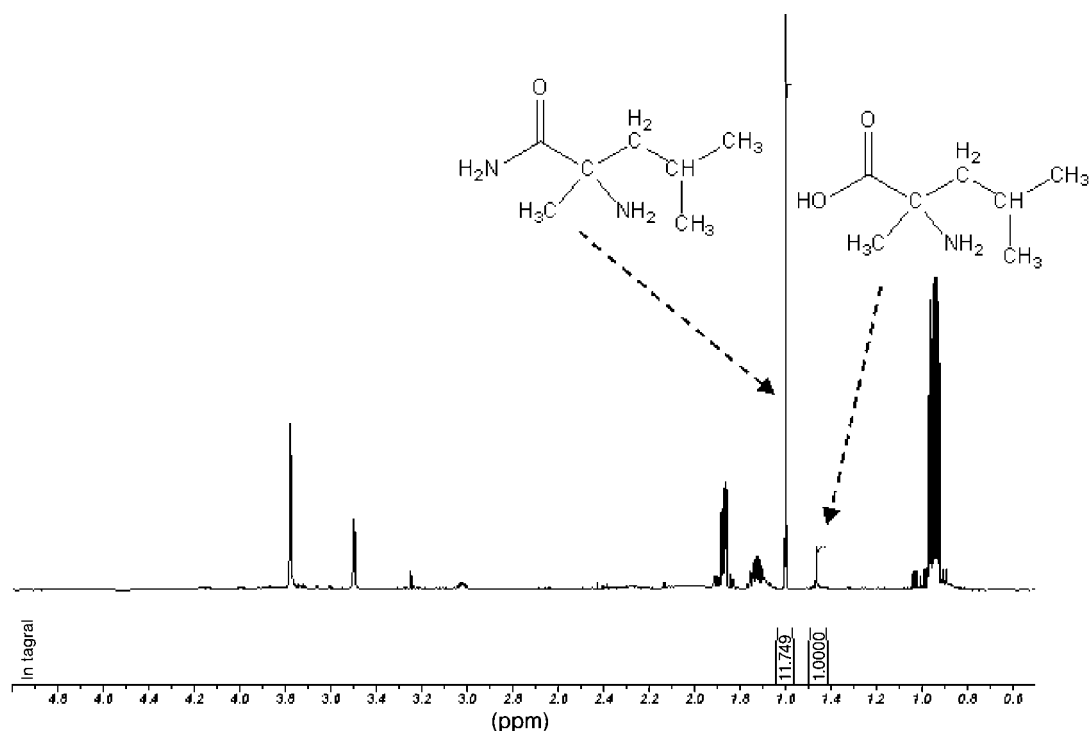


These disubstituted amino acids can be produced in enantiomerically pure form by an amidase process that is quite similar to the process for the production of  $\alpha$ -H- $\alpha$ -amino acids [30]. In this case, however, ketones are used as starting material instead of aldehydes and, since the L-aminopeptidase from *P. putida* ATCC 12633 is not active for this class of amides, a different amidase is applied in the resolution step [30].

The screening assay presented here was intended to identify new amidases with activity towards  $\alpha,\alpha$ -disubstituted  $\alpha$ -amino acid amides. For this purpose,  $\alpha$ -methyl leucine amide (17b) was used as substrate, which will be converted into  $\alpha$ -methyl leucine (18b) by positive clones (Figure 4.15). The actual gene library screening was performed as follows.

#### Procedure 4.5: Amidase Screening by NMR

96-Well microtiter plates filled with rich medium (200  $\mu$ L per well) supplemented with the appropriate antibiotic, were inoculated with transformants from a bacterial gene library in *E. coli*. After incubation for 48 h at 22 °C at a shaking speed of 600 rpm, 100  $\mu$ L of culture was transferred from each well to a wide well plate containing 300  $\mu$ L DL- $\alpha$ -methyl leucine amide (67.5 mM, pH 8.0) and incubated for 24 h at 40 °C. Subsequently, the microtiter plates were centrifuged for 10 min at 1000 $\times$ g to pellet the cells. From the supernatant in each well, 300  $\mu$ L was transferred to deep-well 96-well microtiter plates each containing 50  $\mu$ L D<sub>2</sub>O with 40 g L<sup>-1</sup> maleic acid, 20 g L<sup>-1</sup> EDTA, and 1 g L<sup>-1</sup> DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt) (pH of this solution was adjusted to 6.5). <sup>1</sup>H-NMR measurements were performed at 500 MHz on a Bruker Avance spectrometer using a 4 mm flow-cell (active volume 120  $\mu$ L) equipped with standard Bruker software (XWINNMR version 3.1 and ICONNMR 2.6). Aspiration and dispensing speed were set to 8 mL min<sup>-1</sup> and the sample volume was 300  $\mu$ L. The total measurement time was 25 s (eight scans with two dummy scans), equalling the simultaneous preparation time of the next sample. The overall repetition time was about 60 s. Processing (Fourier transformation, phase correction, baseline correction, and integration) was done per microtiter plate, using parameters optimized for the first sample of the microtiter plate. After a quick visual check of the spectra, the integral values were exported to a text file, which was imported into an Excel file, designed to cal-



**Fig. 4.17**  $^1\text{H}$ -NMR spectrum of a positive hit in the screening for an amidase that hydrolyzes DL- $\alpha$ -methyl leucine amide.

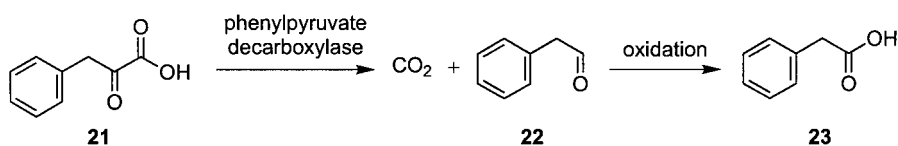
culate the conversions. For each microtiter plate, conversion figures and graphical representation of the results were displayed and plotted.

In total 120 microtiter plates (11 520 samples) were screened in this way, which led to the identification of two positive clones (see also Section 4.4.2.1). A spectrum of one of these positive clones is given in Figure 4.17.

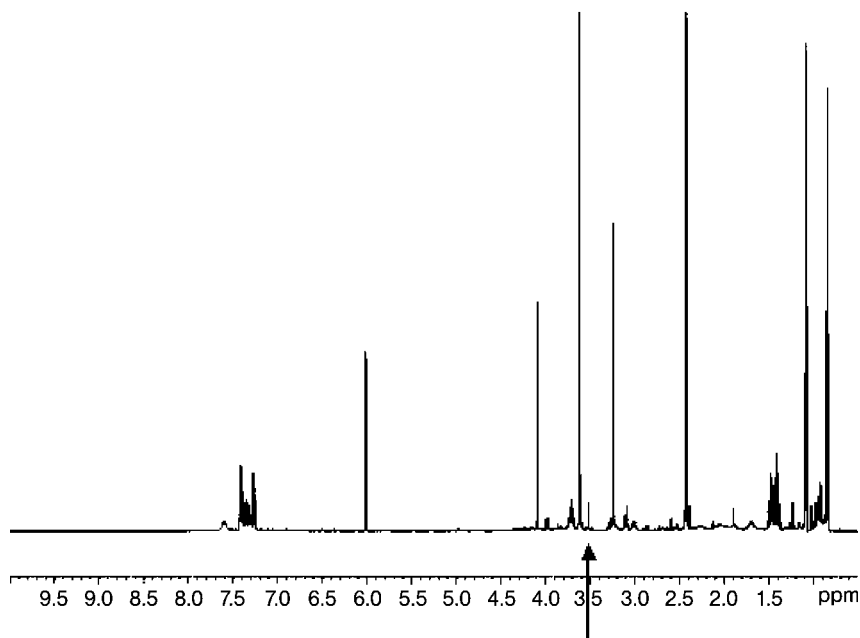
#### 4.4.1.5 Example: Identification of a Phenylpyruvate Decarboxylase Clone

Phenylpyruvate decarboxylases catalyze the decarboxylation of phenylpyruvate (21) resulting in the formation of phenylacetaldehyde (22) (Figure 4.18).

Interestingly, this enzyme can also catalyze the asymmetric acyloin condensation of phenylpyruvate with various aldehydes [51, 52]. In a similar set-up as



**Fig. 4.18** Natural reaction catalyzed by the enzyme phenylpyruvate decarboxylase.



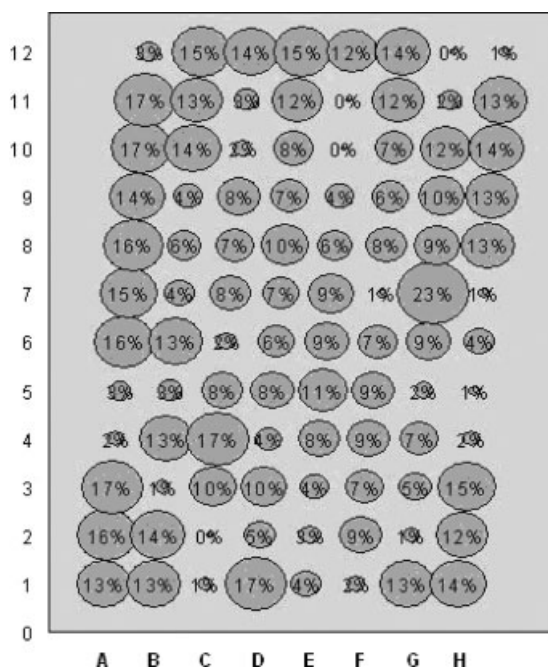
**Fig. 4.19** Spectrum of a positive hit in the phenylpyruvate decarboxylase screening. This spectrum indicates the formation of phenylacetic acid (resonance indicated by the arrow) rather than phenylacetaldehyde, most likely because of (chemical) oxidation of the screening product formed.

that used in the amidase screening described in Section 4.4.1.4, but with 120 mM phenylpyruvate as substrate, a bacterial gene library in *E. coli* was screened and assayed with flow-injection NMR to isolate the phenylpyruvate decarboxylase gene from this microorganism. On visual inspection of the spectra it turned out that there were no hits, as formation of phenylacetaldehyde could be demonstrated in none of the wells. However, a few spectra could be found where the oxidation product of this compound (phenylacetic acid, **23**) was detected (Figure 4.19). Apparently, the primary aldehyde product had been oxidized into the carboxylic acid. Further examination of these clones by nucleotide sequence determination followed by subcloning proved that these hits did indeed contain the desired phenylpyruvate decarboxylase gene.

#### 4.4.1.6 Example: Identification of Amidase Mutants with Improved Activity towards $\alpha$ -Methylphenylglycine Amide

Using a combination of different directed evolution techniques such as epPCR and saturated mutation primer PCR DNA shuffling [53], the specific activity of the L-amidase from *Ochrobactrum anthropi* NCIMB 40321 towards DL- $\alpha$ -methylphenylglycine amide (**17c**, Figure 4.15) could be improved by approximately





**Fig. 4.20** Graphical representation of the results of one microtiter plate obtained in the screening for an amidase with improved activity towards  $\alpha$ -methylphenylglycine amide (expressed as % conversion).

500%. Also in this case the screening method used was identical to that in example 4.4.1.4, now using DL- $\alpha$ -methylphenylglycine amide as substrate. In Figure 4.20 a graphical representation of the results of a typical microtiter plate is given.

#### 4.4.2

##### Fast LC/MS for High-throughput Screening of Enzymatic Activity

Among the techniques developed for high-throughput screening, mass spectrometry offers good potential to act as a generic screening method and has gained increased attention in the past decade [54, 55]. Some high-throughput MS screening methods for biocatalysts have been described, without combination with chromatography. For example MALDI MS methods have been developed and optimized to quantify low-molecular-mass substrates and products of enzyme-catalyzed reactions [56, 57]. Methods based on electrospray ionization mass spectrometry (ESI MS) have been developed for the determination of conversion [58] and enantioselectivity [59, 60] of biocatalytic reactions, in which  $10^3$ – $10^4$  biocatalysts can be screened per day. Despite the high throughput that can be realized by the above-mentioned flow-injection analysis (FIA) MS systems, by-products may not be detected in this mode [61] and screening for

enantioselective (bio)catalysts necessitates the use (and consequently the synthesis) of *pseudo*-enantiomers (chiral compounds differing in absolute configuration and isotopic labeling only) or *pseudo*-prochiral compounds [59]. The development of simple, reliable LC/MS interfaces, most notably ESI and atmospheric pressure chemical ionization (APCI), has spurred the development and acceptance of LC/MS methods, such that, nowadays, there are many laboratories that routinely use LC/MS as the primary analytical method.

The LC system is used to fractionate a preparation and the mass spectrometer is positioned as a detector, monitoring the effluent. With regard to fast LC/MS methods for the analysis of small molecules (mol wt <1000) in biological matrices, an overview of these methods has recently been published [62]. In this review article, fast LC/MS methods for analytes in, for example, blood, plasma, serum, urine, and saliva, are described. Both gradient and isocratic methods are discussed, whereby 'fast' is defined as exhibiting a cycle time of less than 5 min. For the determination of enantioselectivity, fast chiral HPLC/MS methods are reported using commercial chiral stationary phases (CSP) [63–65]. Despite the fact that numerous LC/MS applications for blood and plasma are described in the literature, relatively few studies deal with LC/MS applications for medium to high-throughput screening of biocatalysts [66, 67].

Because of its sensitivity, specificity, and medium to high throughput capability, LC/MS is often the method of choice for screening unpurified biotransformations where low conversion numbers are expected. The throughput required with a large "random" screening population dictates for both the HPLC and mass spectrometer a specific set-up. Fast chromatographic run times per sample are required, which necessitate the use of short column lengths, accompanied by sufficient efficiency. The latter may be realized by using either particulate column packings with small particles (for instance 2 or 3  $\mu\text{m}$ ) or silica rods. With respect to the mass spectrometric part, both LC/MS and LC/MS/MS can be run in both positive and negative ion modes.

#### 4.4.2.1 Example: Screening of a Bacterial Expression Library for Amidase-containing Clones [68]

This LC/MS biocatalyst screening was set up in our laboratory for validation of the hits found with the flow-injection NMR screening assay described in Section 4.4.1.4. The screening assay was intended to determine amidase activity towards  $\alpha$ -methyl leucine amide (**17b**, Figure 4.15) as example for  $\alpha,\alpha$ -disubstituted  $\alpha$ -amino acid amides, so the product of this reaction was  $\alpha$ -methyl leucine (**18b**, Figure 4.15).

#### Procedure 4.6: LC/MS Assay of Amidase Activity

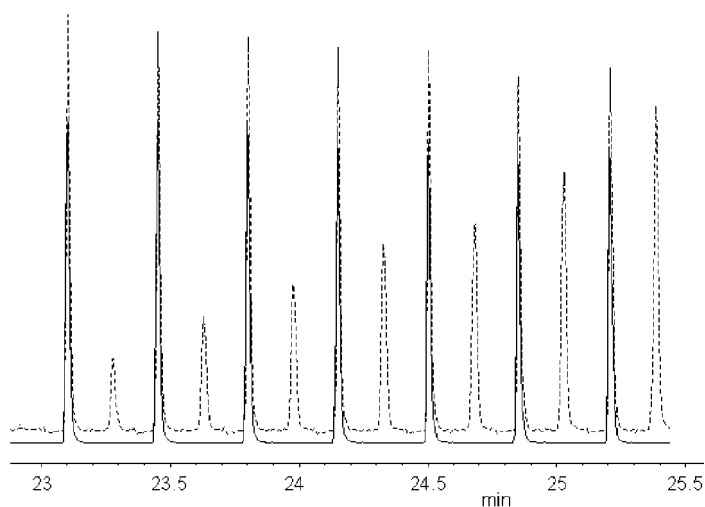
The two 96-well plates out of 120 containing hits according to the flow-injection NMR screening assay, were centrifuged for 10 min at  $1000\times g$ . From the supernatant (cleared reaction mixture) in each well, 5  $\mu\text{L}$  was trans-

ferred to shallow 96-well microtiter plates each containing 245  $\mu\text{L}$  acetonitrile/water (50/50) supplemented with  $\text{NH}_4\text{OH}$  (0.5 weight %, pH 10.8). The LC/MS system used for the screening of the two plates consisted of an Agilent 1100 system with a binary pump, a well-plate sampler with hand-held controller and an Agilent 1100 mass-selective detector (MSD). For HPLC, a  $50 \times 4.6$  mm SpeedRod column (Merck) was used in combination with an isocratic mobile phase (1.5 weight % acetonitrile in 0.1% formic acid at a flow-rate of  $4 \text{ mL min}^{-1}$  at room temperature). This system operates well with 0.3–1  $\mu\text{L}$  injections and a whole-plate injection program.

Analysis time per well is 20 s, resulting in a cycle time of 32 min for a 96-well plate. The mass spectrometer was operated in the selected ion mode (SIM) at  $m/z$  146, that is the  $[\text{M}+\text{H}]^+$  ion of the acid, and at  $m/z$  145, that is the  $[\text{M}+\text{H}]^+$  ion of the acid amide.

To ensure quantitative LC/MS analysis, use of a complementary calibration procedure as described in Section 4.3.2.1 is essential. The complementary calibration mixtures were prepared by mixing a solution of DL- $\alpha$ -methyl leucine amide HCl (67.5 mM) and a solution of L- $\alpha$ -methyl leucine and  $\text{NH}_4\text{OH}$  (67.5 mM each) in increasing ratios covering a range of 0–2.54 mM L- $\alpha$ -methyl leucine (corresponding to 0–3.75% conversion of the L- $\alpha$ -methyl leucine amide substrate). The starting concentration of the amino acid amide in the 0% conversion calibration sample corresponds to the amount of amino acid amide used as substrate in the screening experiments. A conversion of 50% means that all of the L-amino acid amide is converted into L-amino acid and ammonia. As solvent for both solutions, the same rich medium as applied in the screening protocol was used in which *E. coli* had been cultured over 24 h at  $20^\circ\text{C}$ . After removing the cells by centrifugation, the supernatant was diluted 4 times with Milli-Q water (Millipore, Bedford, MA, USA) before dissolving the calibration curve components. The chromatograms obtained for the calibration solutions by SIM are given in Figure 4.21.

In all calibrations carried out during the LC/MS screening assay, linear regression analysis indicated that the correlation coefficient of each single calibration curve was better than 0.994. The coefficient of variation for seven calibrations was of the order of 5%. The detection limit of conversion that could be determined was about 0.1% conversion in the case of  $\alpha$ -methyl leucine amide. For quantitation, the ratio of the acid and the amide (positive ion detection) was taken. By this method of internal standardization, the effect of possible evaporation is corrected. The two hits identified by flow-injection NMR were also found by LC/MS assay. The results are shown in Table 4.2. The percentage conversion found by using LC/MS was somewhat lower than those measured by flow-injection NMR, however, in both assays the two positive hits could be clearly identified among the whole sample population.



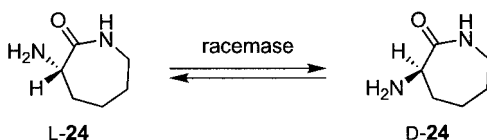
**Fig. 4.21** Chromatograms of complementary calibration mixtures recorded in SIM mode ( $m/z$  145 is  $[M+H]^+$  of  $\alpha$ -methyl leucine amide (black line);  $m/z$  146 is  $[M+H]^+$  of  $\alpha$ -methyl leucine (dashed line)). The abundance plots are normalized to full scale.

**Table 4.2** Amidase hit identification (expressed as % conversion of DL- $\alpha$ -methyl leucine amide) in a bacterial gene library by flow-injection analysis NMR (FIA NMR) and LC/MS.

Plate number	Well number	FIA NMR (% conv.)	LC/MS (% conv.)
77	E4	3.7	3.3
97	B5	3.4	2.2

#### 4.4.2.2 Example: Screening of Enzymatic Racemase Activity [66]

As a second example we describe the use of LC/MS for the screening of enzymatic racemase activity. In this specific case, clones from an expression library were screened for a racemase for  $\alpha$ -amino- $\epsilon$ -caprolactam (ACL, **24**) using a fast chiral LC separation method combined with ion-spray MS as the detection technique. The determination of the conversion in a racemase reaction necessitates



**Fig. 4.22** Reaction catalyzed by an  $\alpha$ -amino- $\epsilon$ -caprolactam (ACL) racemase.

the use of a method to distinguish both enantiomers of the substance, as one enantiomer serves as substrate for the enzyme, while the other is the product of the reaction (Figure 4.22).

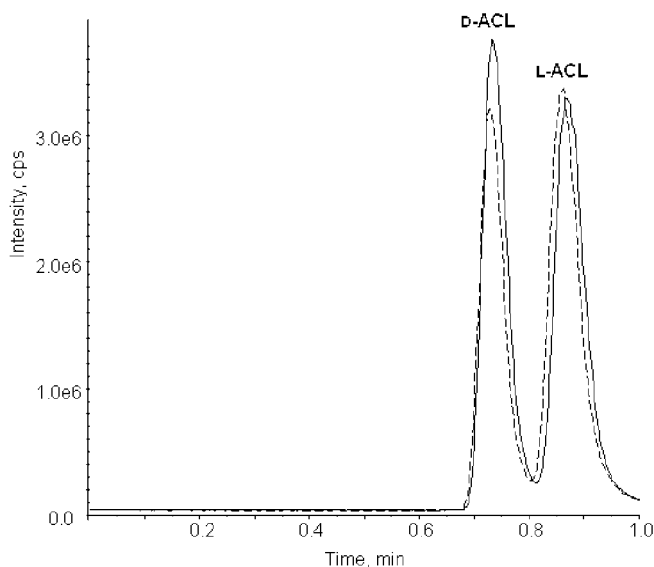
#### Procedure 4.7: LC/MS Assay for Racemase

Using a Crownpak CR (+) column and an aqueous perchloric acid solution as mobile phase, baseline separation of the ACL (24) enantiomers was obtained. Since perchloric acid is a nonvolatile acid, this type of mobile phase is not suited for interfacing with a mass spectrometer.

To circumvent this problem, the mineral acid may be substituted by a short-chain organic acid as mobile phase additive. In the literature, the use of trifluoroacetic acid and acetic acid as mobile phases for Crownpak columns has been reported. In our case it is necessary to protonate the ACL enantiomers in order to obtain sufficient retention and resolution on the crownether stationary phase. As the  $pK_a$  of ACL is 7.8, an acidic pH is necessary to transform the enantiomers into their ammonium form. A 0.1% aqueous solution of formic acid (measured pH 2.6) was chosen as eluent instead of the nonvolatile perchloric acid solution. The enantioselectivity and resolution found by using the formic acid eluent were comparable with those of the perchloric acid mobile phase. By setting the flow-rate to  $1.5 \text{ mL min}^{-1}$ , a run time of 1 min per sample was obtained. Using overlapping injections, a total analysis time of 56 min per 96-well microtiter plate could be achieved. With respect to the LC/MS interface, the suitability of both the turbo ion spray and the heated nebulizer interface for the ionization of ACL were evaluated. It turned out that the sensitivity for ACL was of the same order of magnitude for both interfaces. However, in the heated nebulizer interface severe peak broadening occurred, which resulted in overlapping peaks of the enantiomers at a flow-rate of  $1.5 \text{ mL min}^{-1}$ . As peak broadening in the heated nebulizer interface is flow-dependent, flow splitting was tried. At lower flow rates the peak broadening improved, but sensitivity was lost as this type of interface is a mass-sensitive interface.

It was noticed that peak broadening in the turbo ion-spray interface was minimal and that the peak separation obtained by LC/UV could be maintained in this MS interface. Ion-spray MS in the positive ion mode was therefore chosen as detection technique for measurement of the  $[M+H]^+$  ion of the ACL enantiomers at  $m/z$  129. The D-ACL enantiomer showed hardly any retention on the column and coeluted with components arising from the biological matrix. This coelution caused some ionization suppression of the signal of the D-ACL enantiomer. The  $m/z$  129 signal showed no contribution from the biological matrix. Figure 4.23 shows selected ion chromatograms of racemic ACL with and without a biological matrix.

The signal for the D-enantiomer was suppressed by about 20%. On the other hand, the L-enantiomer was hardly influenced by the biological ma-

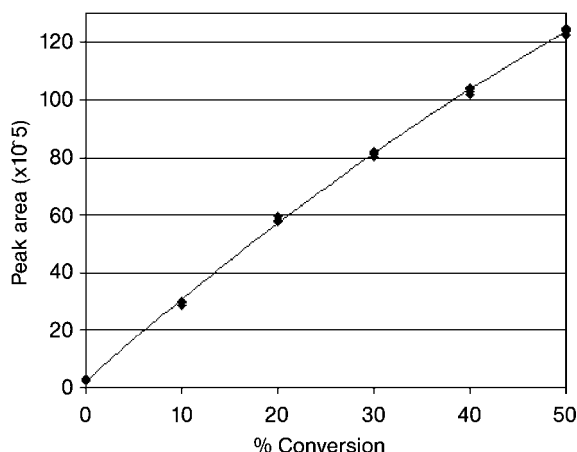


**Fig. 4.23** Selected ion chromatograms ( $m/z$  129) showing the influence of the biological matrix on the signal intensity of the both ACL enantiomers. Chromatogram (black line) showing the analysis of a racemic mixture of ACL without biological matrix and chromatogram (dashed line) with biological matrix.

(Reprinted from *Journal of Chromatography A*, Vol. 1020, H.J.W. Henderickx, A.L.L. Duchateau, P.C. Raemakers-Franken, Chiral liquid chromatography-mass spectrometry for high-throughput screening of enzymatic racemase activity, pp. 69–74, Copyright (2003), with permission from Elsevier).

trix. As the goal of the assay was the measurement of the product of the racemase reaction, that is the D-ACL enantiomer, the difference in suppression between the two enantiomers was not a problem. In the preparation of the calibration standards, a racemase reaction was mimicked in a biological matrix that was comparable with the matrix of the expression library samples. The concentration of the calibration samples covered the range of 0–50% conversion of L-ACL, with 50% conversion implying the presence of equal amounts of D-ACL and L-ACL. Figure 4.24 shows a typical intra-day calibration graph, which was measured in quadruplicate every day.

The nonlinearity of the calibration curve is caused by the limited dynamic range of ion-spray ionization. Ionization suppression and ion signal saturation account for this effect [69]. For the calibrated concentration range, the intra-day coefficient of variation (four replicate injections of the calibration samples) was typically around 8%. The inter-day coefficient of variation was about 15%. The sensitivity of the method allowed a theoretical detection limit of 0.1% conversion. In practice this level could not be attained as the substrate of the reaction already contained 0.5% D-ACL. Because of this impurity of the substrate, the detection limit for the actual screening was set to 1% conversion.



**Fig. 4.24** Typical intra-day calibration graph for D-ACL, showing four replicate data points per calibration level. Optimal fit is obtained with a quadratic curve  $y = -0.0106x^2 + 2.9546x + 2.3098$ . The coefficient of regression is 0.9992.

(Reprinted from *Journal of Chromatography A*, Vol. 1020, H. J. W. Henderickx, A. L. L. Duchateau, P. C. Raemakers-Franken, Chiral liquid chromatography-mass spectrometry for high-throughput screening of enzymatic racemase activity, pp. 69–74, Copyright (2003), with permission from Elsevier).

In total almost 12 000 clones from an expression library were analyzed on a single Crownpak column. No significant change in column performance was noticed after screening the total expression library.

## 4.5

### Conclusions

Although timelines in chemical custom manufacturing are extremely short, the two approaches outlined in this chapter will enable on-time identification of new or improved biocatalysts. The first approach requires proactive development of assay methods that are normally not suitable for this demanding business field because of their long development time. This situation is typical for reader assays requiring set-up of chemical derivatization or enzymatic cascade reactions. Such chemical or enzymatic follow-up reactions are needed, as it is essential to assay the truly desired industrial conversion instead of using artificial colorimetric or fluorometric substrates. The second approach is based on use of instrumental assay techniques such as NMR and MS. As these kind of techniques are very broadly applicable (“generic”), leading to rather short development times, the CCM timelines can be met without any proactive development. We are convinced that by these two screening approaches one of the major hurdles for further implementation of biocatalysis in CCM, in many cases offering the cheapest and most environmentally benign solution, can be cleared.

## Acknowledgments

The authors want to gratefully acknowledge Pieter Grijpstra (BioExplore), Rob van Blijswijk, Gabriëlle v. Bruggen, Richard Kerkman, Ruud Luiten, and Charlotte van Winden (DSM Anti-Infectives, DAI Innovative), Paul Groen and Marjan Zeeman (DSM Food Specialties, Analysis), and Friso van Assema, Math Boesten, Sandra Ernste, Miriam Hillemans-Crombach, Ilse Maes, Wilco Peeters, Elly Raemakers-Franken and Anja Wagemans (DSM Pharma Chemicals, Advanced Synthesis, Catalysis & Development) for their valuable experimental and theoretical contributions. Ben Dassen and Bernard Kaptein (DSM Pharma Chemicals, Advanced Synthesis, Catalysis & Development) are gratefully acknowledged for their essential organic chemical contribution, and Prof. Lubbert Dijkhuizen (Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen), Roel Bovenberg (DSM Anti-Infectives), Birgit Schulze (DSM Food Specialties) and Marcel Wubbolts (DSM Pharma Chemicals) for their stimulating discussions.

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